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Use of Cold Compounds and High Resolution Mass Spectrometry to Quickly Characterize Drug Uptake in Cryopreserved Human Hepatocytes

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Abstract

GOAL

Using cold compounds, we wanted to present a compelling set of data to show that hepatocytes and HRAM spectrometry can be used to obtain at least screening-quality information with regard to whether a test article is a substrate of the solute carrier uptake transporters.

METHODS

In this study, we assessed the use of cryopreserved hepatocytes for evaluating whether test articles are substrates of one of the OA TPs with the oil filtration method [Parker and Houston, 2008]. Hepatocytes suspended in incubation media containing a test article for up to 3 minutes at both 4ºC and 37ºC were filtered through a layer of silicone oil using centrifugation. The cells in the receiving layer containing 2 N NaOH were disrupted and the clarified solutions were analyzed by an Acquity UPLC coupled to a Waters Q-Tof Premier.

Method development for the test articles was performed in parallel with a generic gradient and no manual tuning (< 1 day). Several test articles were screened for accumulation in the hepatocytes. Also, several compounds were screened for their ability to reduce the uptake of typical probe substrates of OATP1B1, OATP1B3 and OATP2B1, estradiol-17ß-glucuronide and estrone-3-sulfate.

CONCLUSIONS

The use of cryopreserved hepatocytes and high resolution mass spectrometry proved to be an effective combination for characterizing uptake transporters without the use of radiolabeled compounds or time-intensive tandem quadrupole analysis. Methods were simultaneously developed for a wide variety of compounds from different chemical classes. Though many compounds would not fit the generic method and would require additional set-up time, sufficient time is saved to make this workflow suitable for substrate and/or inhibition assays early in the drug discovery process.

Introduction

With the recent knowledge of the importance of transporters in drug disposition and the release of the new draft of the FDA guidance for drug interactions, more emphasis has been placed on characterizing the ability of drug candidates to act as substrates and/or inhibitors of certain membrane transporters. In particular, the guidance recommends that if hepatic or biliary secretion of a drug candidate contributes to at least 25% (or unknown) of overall clearance, substrate studies should be conducted for OATP1B1 and OATP1B3. These organic anion transporting polypeptides are expressed on the sinusoidal membrane of hepatocytes, which are recognized as a useful in vitro model for studying these uptake transporters [Xia et al., 2007].

The oil filtration assay [Parker and Houston, 2008] typically used for studying uptake transport with hepatocytes usually relies on radiolabeled compounds, which often are not available until late in the drug development cycle. Alternatively, LC/MS/MS assays with cold compounds can be used, though the assay development with tandem quadrupole mass spectrometers can be time-consuming.

High-resolution accurate mass (HRAM) spectrometry has been used to enhance throughput for method development in a number of applications including metabolic stability, metabolite profiling, and bioanalysis [Campbell and LeBlanc, 2012]. HRAM spectrometry was used in this study with hepatocytes as a means of characterizing transporter properties of test articles earlier in the drug discovery process.

Materials

InVitroGRO Krebs-Henseleit buffer (KHB) media and InVitroGRO CP media were purchased from In Vitro Technologies. Silicone oil (part# 175633), mineral oil (part# M5904), estrone-3-sulfate (E3S), and valaciclovir were purchased from Sigma Aldrich. Sodium hydroxide pellets were purchased from Calbiochem.

Atazanavir, vincristine and atorvastatin were purchased from Toronto Research Chemicals. Piroxicam and erlotinib were purchased from Santa Cruz Biotechnology. Rosiglitazone was purchased from LKT Labs, Inc. Cryopreserved human hepatocytes were provided by Celsis In Vitro Technologies (lot HKT).

Methods

Transporter substrates at various concentrations were incubated with cryopreserved human hepatocytes (2x105 viable cells) suspended in InVitroGRO KHB for 3 minutes at 37°C and 4°C. The relative uptake was calculated as a fold difference by dividing the average peak response at 37ºC into the average peak response at 4°C. Samples were run in triplicate. Substrate solutions and cell suspensions (2 × 106 viable cells/mL) were prepared separately. Incubations were started by the addition of 50 µL of substrate solution to 100 µL of cell suspension in a 48-well plate. Approximately 20 seconds before the end of 3 minutes, the incubation mixture from each well was transferred to a 0.4 mL centrifuge tube containing 100 µL of filtration oil (5 parts silicone oil and 1 part mineral oil) layered on the top of 100 µL of 2N NaOH. The tubes were centrifuged immediately at 13,000 g for 15 seconds to separate the cells from the free substrate.

The tubes were frozen at -70°C for 30 minutes before cutting in the middle of the oil layer. The bottom section was dropped into a 1.7 mL centrifuge tube. The compound was extracted by first neutralizing the base with 100 µL of 2N HCl followed by the addition of 200 µL of methanol. The resulting mixture was vortex mixed, centrifuged and the supernatant transferred to an autosampler vial for analysis. The LC/MS system was comprised of a Waters Acquity Ultra Performance Liquid Chromatograph (UPLC) with an in-line photodiode array detector coupled to a Micromass Q-Tof Premier mass spectrometer (Waters, Milford, MA). The UPLC was equipped with a binary solvent delivery system (high pressure mixing). The lockmass was leucine enkephalin (m/z 556.2771). The mass spectrometer was equipped with MassLynx (Waters, Milford, MA).

HPLC Conditions

Column:	**Supleco Ascentis Express C18, 100 x 2.1 mm, 2.7 µm**
Guard column:	**None**
Flow rate:	**0.3 mL/min**
Column temperature:	**40°C**
Autosampler temperature:	**5°C**
Injection loop:	**10 µL**
Injection loop:	**10 µL**
Divert valve:	**0.0 - 1.3 min, 10.0 - 12.0 min**
Solvent A:	**0.1% Formic Acid in Water**
Solvent B:	**Methanol**

Gradient

Time (min)	Solvent A	Solvent B	Gradient Setting
0.0 - 0.5	95%	5%	N/A
0.5 - 8.0	Decrease to 5%	Increase to 95%	6
8.0 - 10.0	5%	95%	6
10.0 - 10.1	Increase to 95%	Decrease to 5%	6
10.1 - 12.0	95%	5%	N/A

Mass Spectrometer Details

Parameter	Setting
Source temperature:	**100°C**
Desolvation temperature:	**350°C**
Polarity:	**ESI-/ESI+**
Analyzer:	**V mode**
Cone energy:	**40 V**
Collision energy:	**5 eV**
Scan range:	**100 to 1000 amu**

Structures

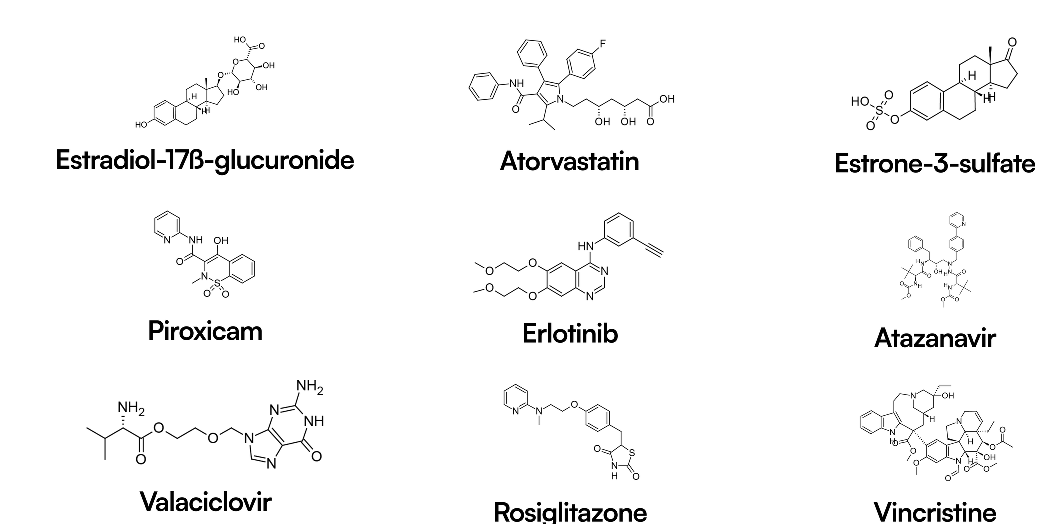


Figure 1

Active Uptake of Various Transporter Substrates into Cryopreserved Human Hepatocytes

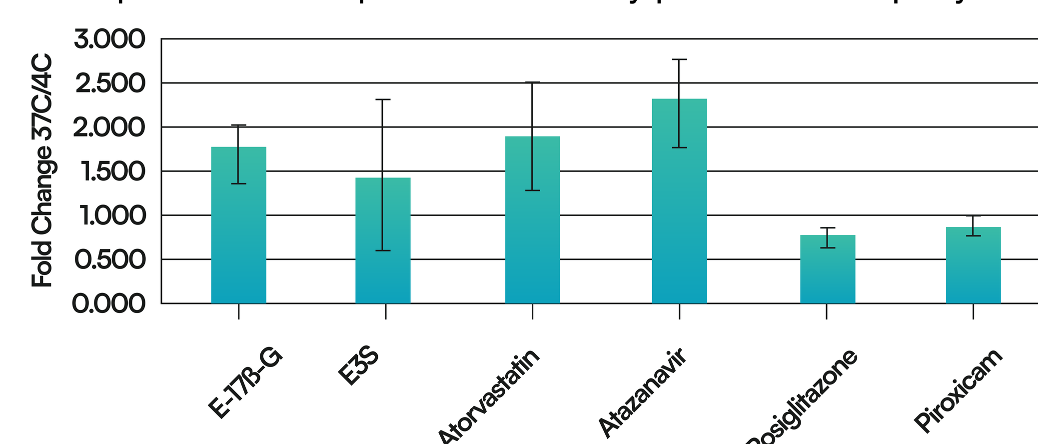


Figure 2

Inhibition of Active Uptake of E-17ß-G into Cryopreserved Human Hepatocytes

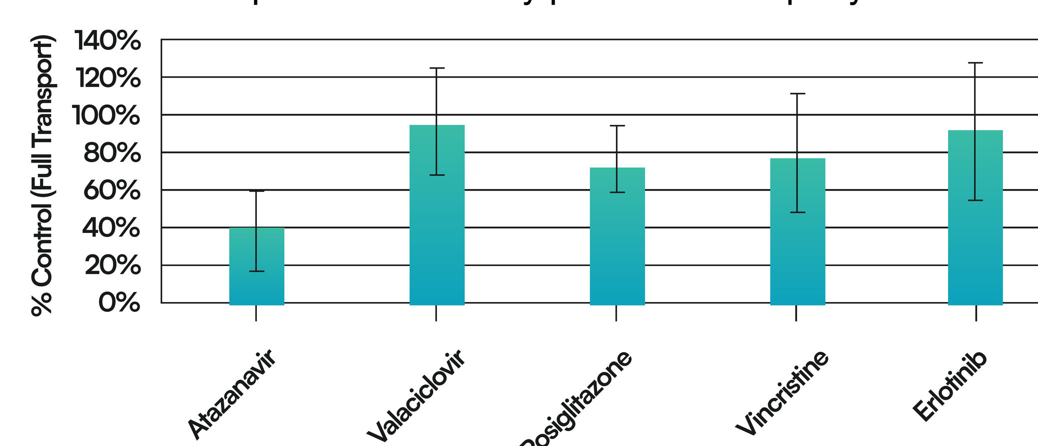
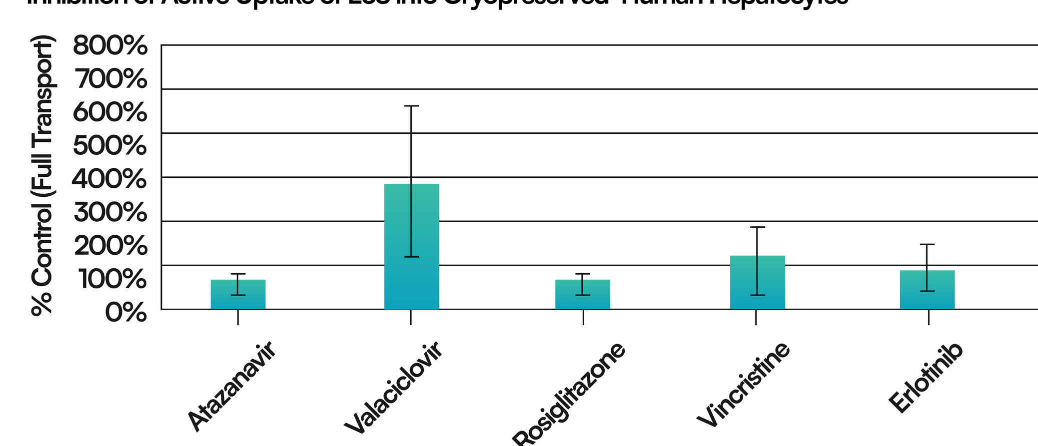


Figure 3

Inhibition of Active Uptake of E3S into Cryopreserved Human Hepatocytes



Results & Discussion

Figure 1 depicts the uptake of various transporter substrates at 10 µM into cryopreserved human hepatocytes.

- E-17ß-G, E3S, atorvastatin, and atazanavir exhibited at least a 1.5-fold increase in accumulation in hepatocytes at 37ºC relative to 4ºC, as expected for these OATP substrates.
- Piroxicam had no measurable active uptake, as expected for a compound exhibiting transcellular uptake.
- Relative intracellular levels of rosiglitazone decreased at 37ºC, suggesting that the known hepatic metabolism of this compound was rapid enough to interfere with interpretation of the uptake assay.

Figure 2 shows the relative change in active uptake of E-17ß-G (1 µM) when co-incubated with various test articles (20 µM).

- Atazanavir strongly inhibited the active uptake of E-17ß-G (< 50% of control), as expected for this OATP inhibitor [Karlgren et al., 2012].
- Rosiglitazone and vincristine exhibited moderate inhibition of active E-17ß-G uptake (50-80% of control), though it was difficult to clearly state the extent of the inhibition due to assay variability.
- Valaciclovir and erlotinib exhibited no inhibition of active uptake of E-17ß-G by hepatocytes. Valaciclovir was a control, and it was not expected to inhibit active uptake. Erlotinib is a fairly specific inhibitor of OATP2B1 [Karlgren et al., 2012], so it was not expected to stop uptake of E-17ß-G by OATP1B1 and OATP1B3.

Figure 2 shows the relative change in active uptake of E-17ß-G (1 µM) when co-incubated with various test articles (20 µM).

- Atazanavir and rosiglitazone potently inhibited the active uptake of E3S (< 50% of control), as expected for these OATP inhibitors [Karlgren et al., 2012].
- Valaciclovir, vincristine and erlotinib did not inhibit the active uptake of E3S. Valaciclovir is a control not expected to inhibit uptake transporters. Vincristine and erlotinib are moderate inhibitors of both OATP1B1 and OATP1B3 [Karlgren et al., 2012]. While erlotinib is a strong inhibitor of OATP2B1 [Karlgren et al., 2012], E3S was still able to enter the hepatocytes through OATP1B1 and OATP1B3.

Conclusions

- Cryopreserved hepatocytes plus HRAM can quickly and effectively screen non-radioactive test articles as:
  - Substrates of active uptake transporters
  - Strong, broad inhibitors of active uptake
- More robust analytical methods seem to be needed to characterize moderate inhibitors due to variability.
- Specific probe substrates for each OATP isoform would help with experimental design interpretation.

References

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